Supplementary Information

Positive regulation of p53 stability and activity by the deubiquitinating enzyme Otubain 1

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Materials and methods

Transfection, immunoblot (IB) and co-immunoprecipitation (Co-IP) analyses. Cells were transfected with plasmids using TransIT®-LT1 reagents following the manufacturer's protocol (Mirus Bio Corporation). Cells were harvested at 36-48 hours posttransfection and lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM DTT, 1 μg/ml pepstatin A, and 1mM leupeptin. Equal amounts of clear cell lysate were used for immunoblot (IB) analysis as previously described (Dai et al, 2008). Co-IP was conducted as described previously (Dai & Lu, 2004; Dai et al, 2008). Bound proteins were detected by IB using antibodies as indicated in figure legends.

Reverse transcriptase-Quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from cells using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA). Reverse transcriptions were performed as described (Dai & Lu, 2004). Quantitative real-time PCR was performed on an ABI StepOne™ real-time PCR system (Applied Biosystems) using SYBR Green Mix (Bio-Rad) as described previously (Sun et al, 2007). All reactions were carried out in triplicate. Relative gene expression was calculated using the $\Delta C\tau$ method following the

manufacturer's instruction. The primers for *p21, mdm2*, *bax*, and *GAPDH* were described (Sun et al, 2010).

Glutathione S-transferase (GST)-fusion protein association assays. His-tagged Otub1 protein was purified from bacteria through a Ni^{2+}-NTA (Qiagen) column. GST-fusion protein-protein association assays were conducted as described (Dai, et al. 2008). Briefly, purified His-Otub1 proteins (200 ng) were incubated with the glutathione-Sepharose 4B beads (Sigma) containing 200 ng of GST-p53, GST-MDM2, or GST alone, respectively. After wash, bound proteins were analyzed using IB with anti-Otub1 antibodies.

Cell Fractionation. U2OS cells treated with or without DNA damaging agents were resuspended in a hypotonic buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) and homogenized. After centrifugation, the supernatant was collected as the cytoplasmic fraction. The pellets were resuspended in buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 25% Glycerol) and sonicated. The nuclear fraction (supernatant) was collected by centrifugation.

***In vivo* ubiquitination assay.** *In vivo* ubiquitination assay was conducted as previously described with minor modification (Dai et al, 2008; Sun et al, 2010). H1299 cells (60% confluence/60mm plate) were transfected with His_6-ubiquitin (1μg), p53 (0.2μg), HA-MDM2 (1μg), or Flag-Otub1 (1 μg) expression plasmids using TransIT®-LT1 reagents (Mirus). The cells were treated with 40 μM MG132 for 6 hour before harvesting. The cells were harvested at 48 hours after transfection and 20% of the cells were used for direct IB and the rest of cells were used for ubiquitination assays under denaturing conditions using Ni^{2+}-NTA

pulldown. The bead bound proteins were analyzed using IB with anti-p53 (DO-1) antibody.

Supplementary Figure Legends

Figure S1. Otub1 stabilizes p53. (A). Overexpression of Otub1 induced p53 in a dose-dependent manner. U2OS cells were transfected with increasing amounts of Flag-Otub1 vector and assayed for the expression of indicated proteins by IB. **(B)**. Dose-dependent effect of Otub1, but not Otub2, in inducing p53. T-Rex-U2OS-Otub1 or T-Rex-U2OS-Otub2 clones were cultured in the presence of doxycycline and harvested at different time points as indicated. The cell lysates were immunoblotted with antibodies as indicated. **(C).** Induction of p53 by Otub1 is comparable to that by USP7. U2OS cells transfected with Otub1, USP7, or control vector were subjected to IB analysis of the indicated proteins. **(D)**. Otub1 induces p53 in H460 cells. p53 wild-type H460 lung cancer cells were transduced with lentiviruses encoding Otub1 or control viruses for 48 hours. The protein expression was analyzed using IB. (E). Overexpression of Otub1 does not increase the levels of *p53* mRNA. U2OS cells transfected with control or Flag-Otub1 plasmid were assayed for *p53* mRNA expression using RT-qPCR. (F) (G). Overexpression of Otub1 increases the half-life of MDM2, but not p21. U2OS cells were transfected with control or Flag-Otub1 plasmid for 48 hours and then treated with 50 μg/ml CHX. The cells were harvested at different time points and assayed for the levels of p21, MDM2, and tubulin by IB (F). The bands were quantified and the levels of p21 and MDM2 were normalized with the levels of tubulin and results from three independent experiments were plotted in G.

Figure S2. **Otub1 stimulates p53 activity and induces p53-dependent cell growth inhibition.** (**A**). Otub1 induces p53 activity. U2OS cells transfected with Otub1, Otub2, or control plasmid were analyzed for the expression of the *p21*, *mdm2* and *bax* mRNA using RT-qPCR analysis and normalized against the expression of *GAPDH*. (**B**). Otub1, but not Otub2, induces apoptosis. T-Rex-U2OS-Flag, T-Rex-U2OS-Flag-Otub1, or T-Rex-U2OS-Flag-Otub2 cells were cultured in medium containing 2μg/ml Dox for 24 hours. The cells were then fixed and stained with PI followed by flow cytometry analysis for the DNA content. The average percentages of sub-G1 phase cells from three-independent experiments are shown in the top panel. (**C)(D**). Otub1 does not induce apoptosis in p53-null H1299 and Saos2 cells. Saos2-tet-Otub1 (C) or H1299-tet-Otub1 (D) cells were cultured in medium containing Dox for 24 hours. The cells were stained with PI and examined for DNA content using flow cytometry analysis. (**E**). Otub1 inhibits cell proliferation. The relative colony number assayed in Fig. 2D was counted from three independent experiments and plotted. (**F**). Otub1 does not inhibit cell proliferation in H1299 cells. Colony formation assays were performed in H1299-tet or H1299-tet-Otub1 cells in the absence or presence of 2 μg/ml Dox. The colonies were visualized by staining with crystal violet blue. (**G**). (**H**). Otub1 inhibits cell proliferation dependent on p53. Colony formation assays were performed in HCT116$^{p53+/+}$-TO-Flag, HCT116$^{p53+/+}$-TO-Flag-Otub1, HCT116$^{p53-/-}$-TO-Flag, or HCT116$^{p53-/-}$-TO-Flag-Otub1 cells in the absence or presence of 2μg/ml Dox for up to 3 weeks. The colonies were visualized by staining with crystal violet blue (G). The relative colony number were counted from three independent experiments and plotted in (H).

Figure. S3. (A). Otub1, but not Otub2, binds to p53. H1299 cells were transfected with p53 with or without Flag-Otub1 or Flag-Otub2. The cell lysates were immunoprecipitated with anti-Flag antibodies followed by IB with polyclonal anti-p53 and anti-Flag antibodies **(B).** Expression of wild-type, but not the deletion mutants, of Otub1 induces p53. U2OS cells were transfected with wild-type or the N-terminus (residues 1-84) and the C-terminus (residues 161-271) of Otub1. The cells were analyzed for the expression of the indicated proteins using IB. **(C)**. Otub1 deubiquitinates p53 to a similar extent as USP7 in cells. H1299 cells were transfected with different combinations of plasmids encoding His-Ub, p53, HA-MDM2, and with or without Flag-Otub1 or Flag-USP7. The cells were subjected to Ni^{2+}-NTA pulldown under denaturing conditions, followed by IB to detect the ubiquitinated species of p53. (D). Otub1 suppresses ARF-BP1-mediated p53 ubiquitination. H1299 cells transfected with indicated plasmids were subjected to *in vivo* ubiquitination assays using Ni^{2+}-NTA pulldown under denaturing conditions, followed by IB to detect the ubiquitinated species of p53. The protein expression was shown in bottom panels.

Figure S4. (**A**). Deletion of residues 88-91 abolishes the Otub1's activity to induce p53. U2OS cells transfected with wild-type Otub1 or its mutant with deletion of residues 88 through 91 ($Otub1^{\Delta 4}$). The cell lysates were examined for the expression of indicated proteins using IB. (**B**). Overexpression of $Otub1^{D88A}$ does not further decrease the levels and activity of p53 by Otub1 knockdown. U2OS cells transfected with scrambled or Otub1 siRNA and control or Flag-$Otub1^{D88A}$ plasmid as indicated. The cells were assayed for the expression of the indicated

proteins using IB. **(C).** Overexpression of the Otub1^{D88A} mutant inhibits p53 activation by treatment with 5-FU. U2OS cells transfected with control or Otub1^{D88A} vector were treated with 5-FU (50 μg/ml) for 8 hours. The cells were then analyzed for the expression of indicated proteins using IB. (**D**). Overexpression of the Otub1^{D88A} mutant inhibits p53 activity following stress. U2OS cells transfected with control or Otub1^{D88A} vector were treated with DNA-damaging agents as indicated for 5 hours. The cells were then subjected to RT-qPCR analysis for the expression of *p21* (top panel) and *mdm2* (bottom panel) mRNA.

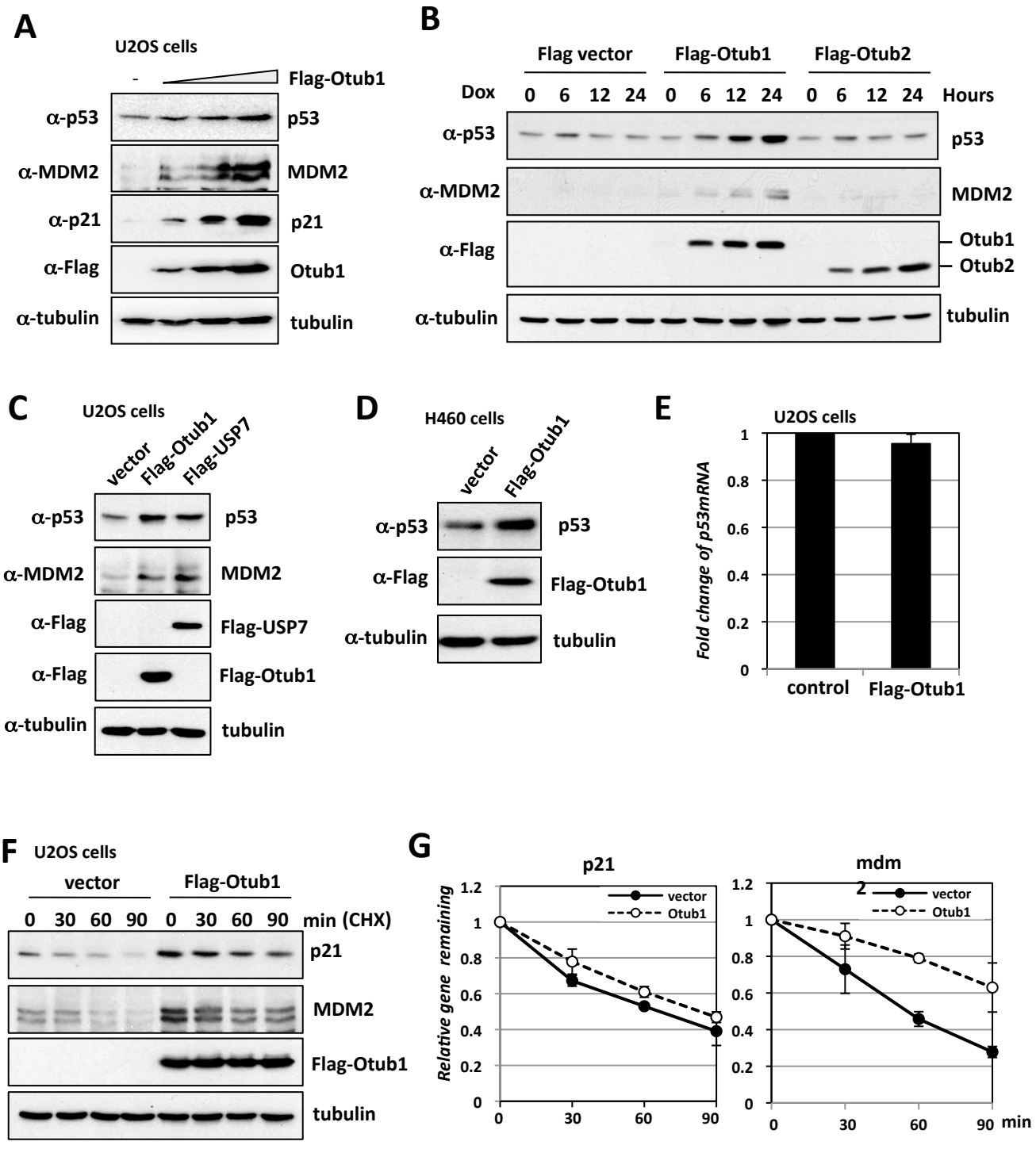
Figure. S5. Otub1 mediates the cleavage of K48-linked polyubiquitin chains and ubiquitinated p53 *in vitro*. (**A**). K48-linked polyubiquitin (Ub_{3-7}) (200 ng, Boston Biochem) was incubated with 1 μM (final concentration) of recombinant wild-type, C91S, or D88A mutants of His-Otub1 proteins purified from bacteria in 20 μl reaction buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM DTT at 37 ^{0}C for 4 hours. The reactions were stopped by boiling in 1X SDS sample buffer and analyzed using IB with anti-ubiquitin and anti-Otub1 antibodies. (**B**). Wild-type Otub1, but not Otub1^{C91S} and Otub1^{D88A} possess weak DUB activity towards polyubiquitinated p53 *in vitro*. Ubiquitinated p53 was purified from H1299 cells transfected with His-Ub, p53, and MDM2 plasmids using anti-Flag affinity purification method. The ubiquitinated p53 was incubated with His-tagged wild-type Otub1, Otub1^{C91S}, and Otub1^{D88A} proteins purified from bacteria in 20 μl reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM DTT at 37 ^{0}C for 4 hours. The reactions were stopped by boiling in 1X SDS sample buffer and analyzed using IB with anti-ubiquitin and anti-Otub1 antibodies.

Figure S6. Otub1 interacts with MDM2 and suppresses MDM2 autoubiquitination. (A). Otub1 interacts with MDM2 in cells. H1299 cells were transfected with Flag-Otub1 individually or together with HA-MDM2. The cell lysates were immunoprecipitated with anti-Flag antibodies followed by IB with anti-MDM2 and anti-Flag antibodies. (**B**) (**C**). Otub1 directly binds to MDM2 *in vitro*. Purified GST or GST-Otub1 immobilized on glutathione beads was incubated with purified His-MDM2 (B). Purified GST or GST-MDM2 immobilized on glutathione beads was incubated with purified His-Otub1 (C). Bound proteins were assayed using IB with anti-MDM2 (B) or anti-Otub1 (C) antibodies. Commassie staining of the GST, GST-Otub1, or GST-MDM2 proteins is shown in the bottom panel of each Figure. (**D**). Co-IP between Otub1, MDM2 and p53. Lysates from H1299 cells transfected with indicated plasmids were immunoprecipitated with anti-Flag antibodies, followed by IB with anti-p53, anti-MDM2, and anti-Otub1 antibodies. The cell lysates were also directly assayed for protein expression as indicated in bottom panels. (**E**). Otub1 suppresses MDM2 autoubiquitination. H1299 cells transfected with indicated plasmids were subjected to *in vivo* ubiquitination assays using Ni^{2+}-NTA pulldown under denaturing conditions, followed by IB to detect the ubiquitinated species of MDM2. The protein expression was shown in bottom panels. (**F**). Otub1 stabilizes MDM2. H1299 cells transfected with indicated plasmids were assayed for protein expression as indicated by IB. (**G**). DNA damage increases the interaction of Otub1 with MDM2 and p53 in cells. RKO cells treated without or with Eto (20 μM) for 5 or 8 hours were subjected to co-IP with purified polyclonal anti-Otub1 antibodies followed by IB.

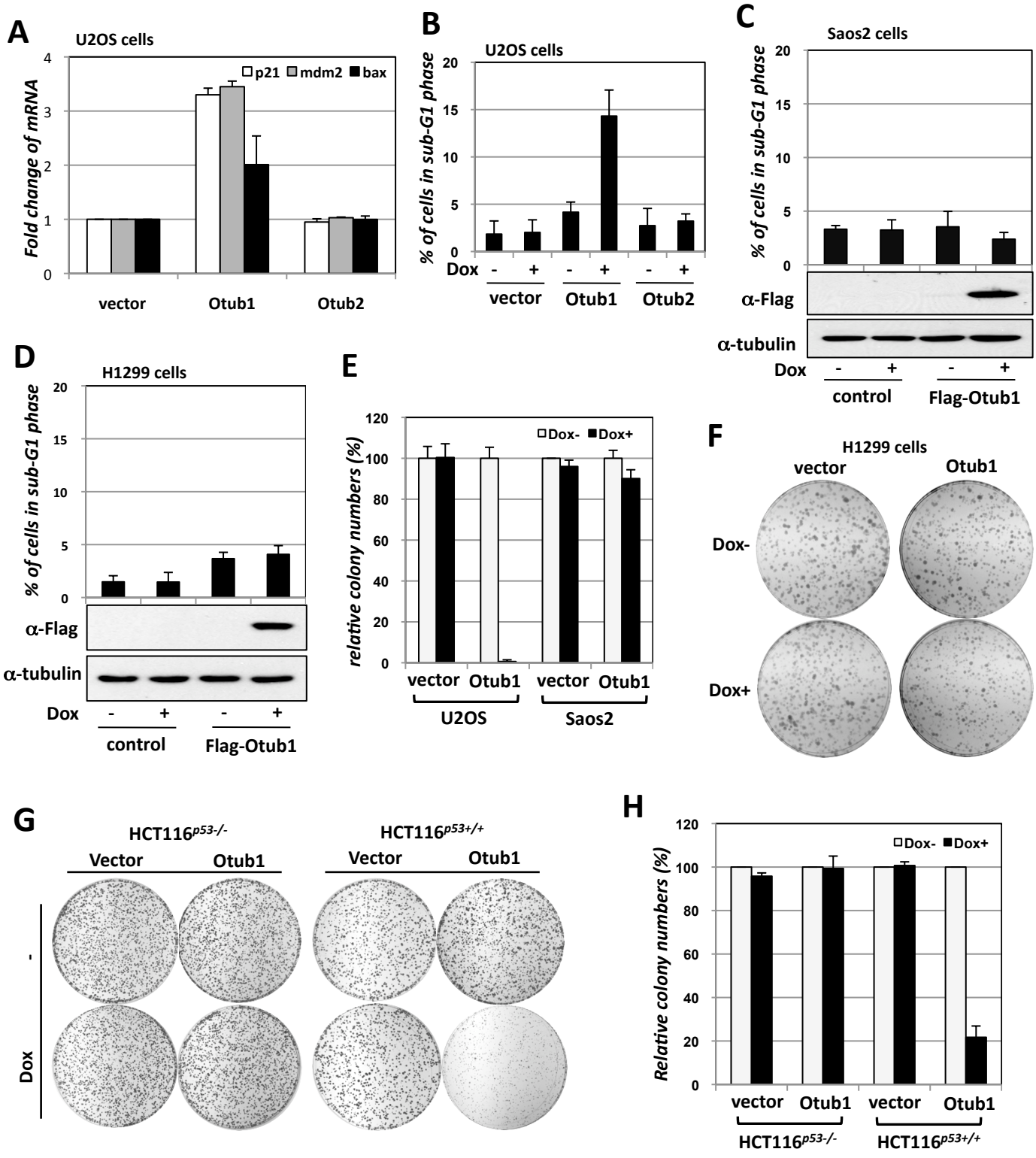
Fig. S7. Otub1 interacts with UbcH5 in cells and suppresses its activity. (A). Dose-dependent suppression of MDM2-mediated p53 ubiquitination by Otub1 *in vitro*. The *in vitro* ubiquitination assay was conducted as in Fig. 7D in the absence or presence of indicated amounts of wt His-Otub1 or His-Otub1^{C91S}, followed by IB with anti-conjugated Ub antibody (Clone FK2) (top panel), anti-Otub1 and anti-UbcH5 antibodies (bottom panel). (**B**)-(**D**). H1299 cells were transfected with V5-tagged UbcH5a (B), UbcH5b (C), or UbcH5c (D) individually or together with Flag-tagged wild-type Otub1 or its C91S or D88A mutant. The cell lysates were immunoprecipitated with anti-Flag antibodies followed by IB with anti-V5 and anti-Flag antibodies.

Fig. S8. (A). DNA damage increases the interaction between p53 and Otub1 in RKO cells. RKO cells were treated with DMSO or Eto for 5 hours. Cell lysates were immunoprecipitated with polyclonal anti-Otub1 antibodies or pre-immune IgG followed by IB with anti-p53 (DO-1) and anti-Otub1 antibodies. (**B**). Otub1 is localized in the cytoplasm. RKO or WI38 cells were fractionated into the cytoplasmic (C) and nuclear (N) fractions followed by IB detection of the indicated proteins. (**C**). Otub1 interacts with p53 in the cytoplasm in response to DNA damage. U2OS cells treated with Eto (20 μM) were harvested at different time points followed by cell fractionation. The cytosolic lysates were immunoprecipitated with anti-Otub1 antibodies followed by IB with anti-p53 antibodies.

Supplementary Figure S1



Supplementary Figure S2



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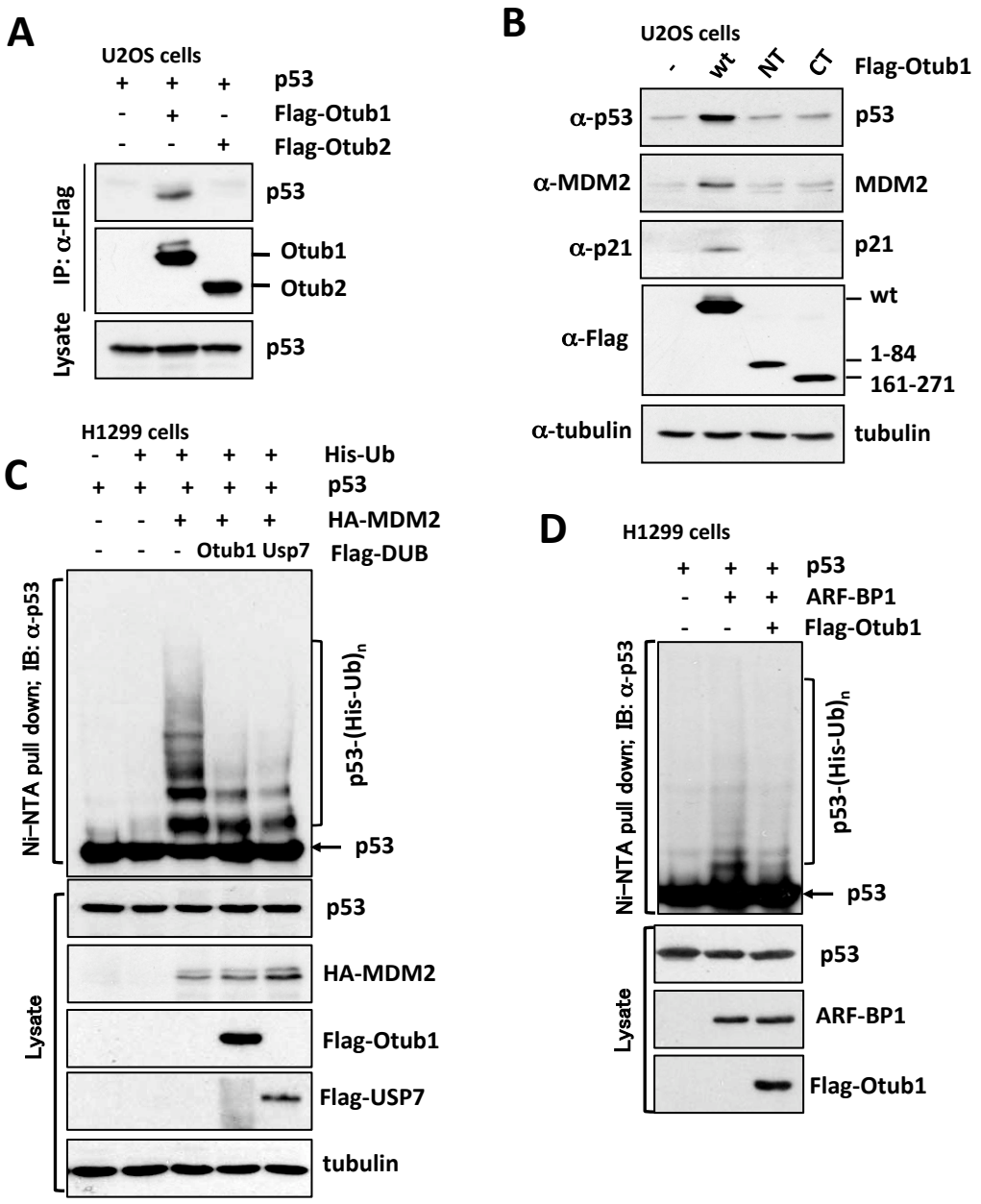

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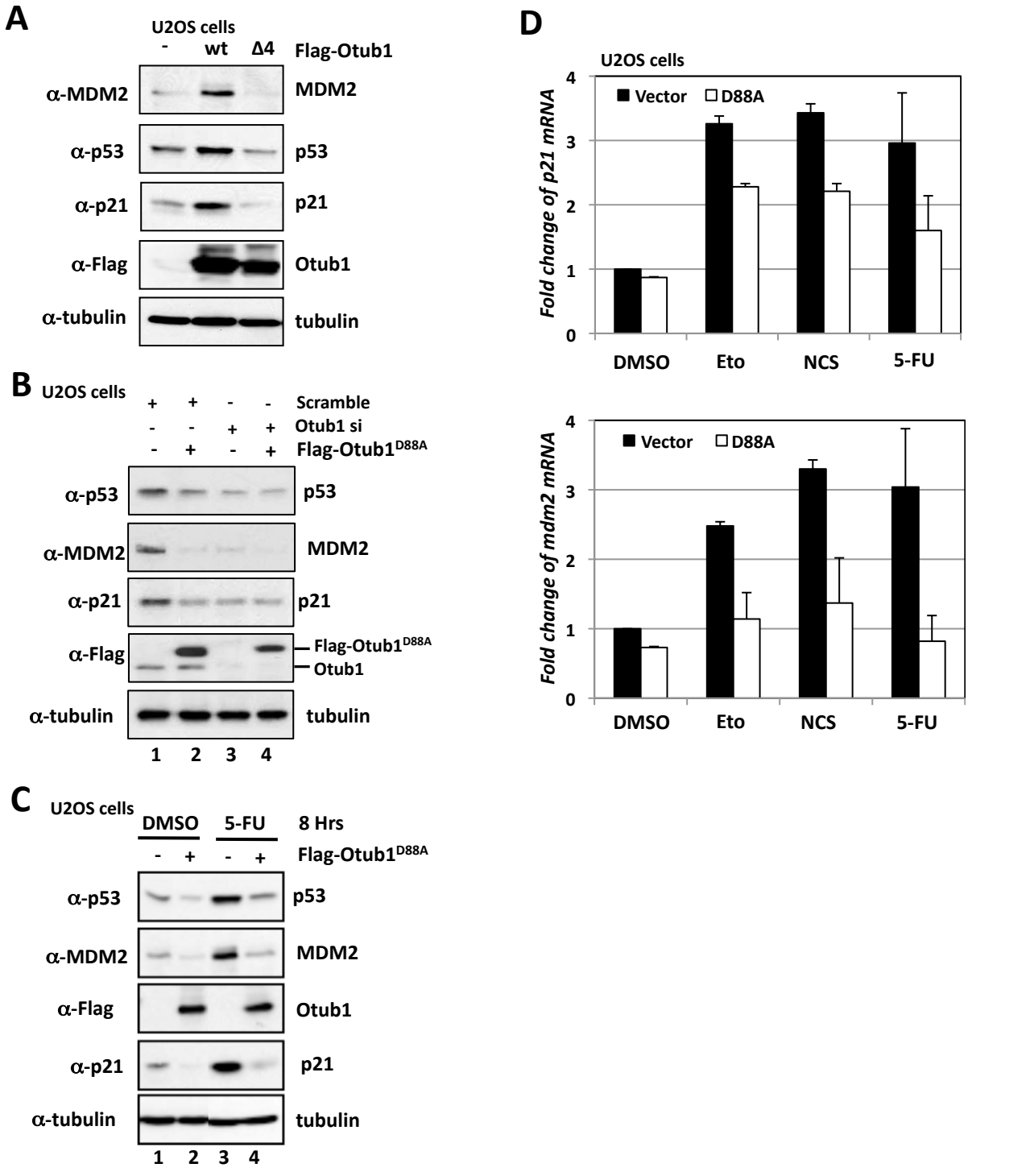

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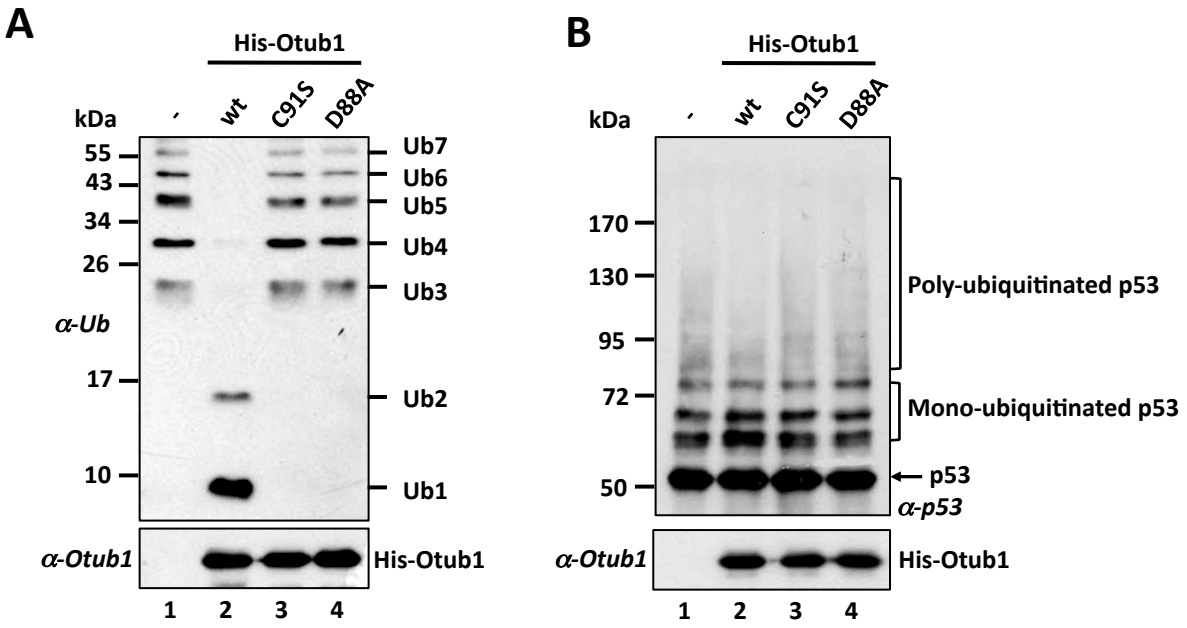

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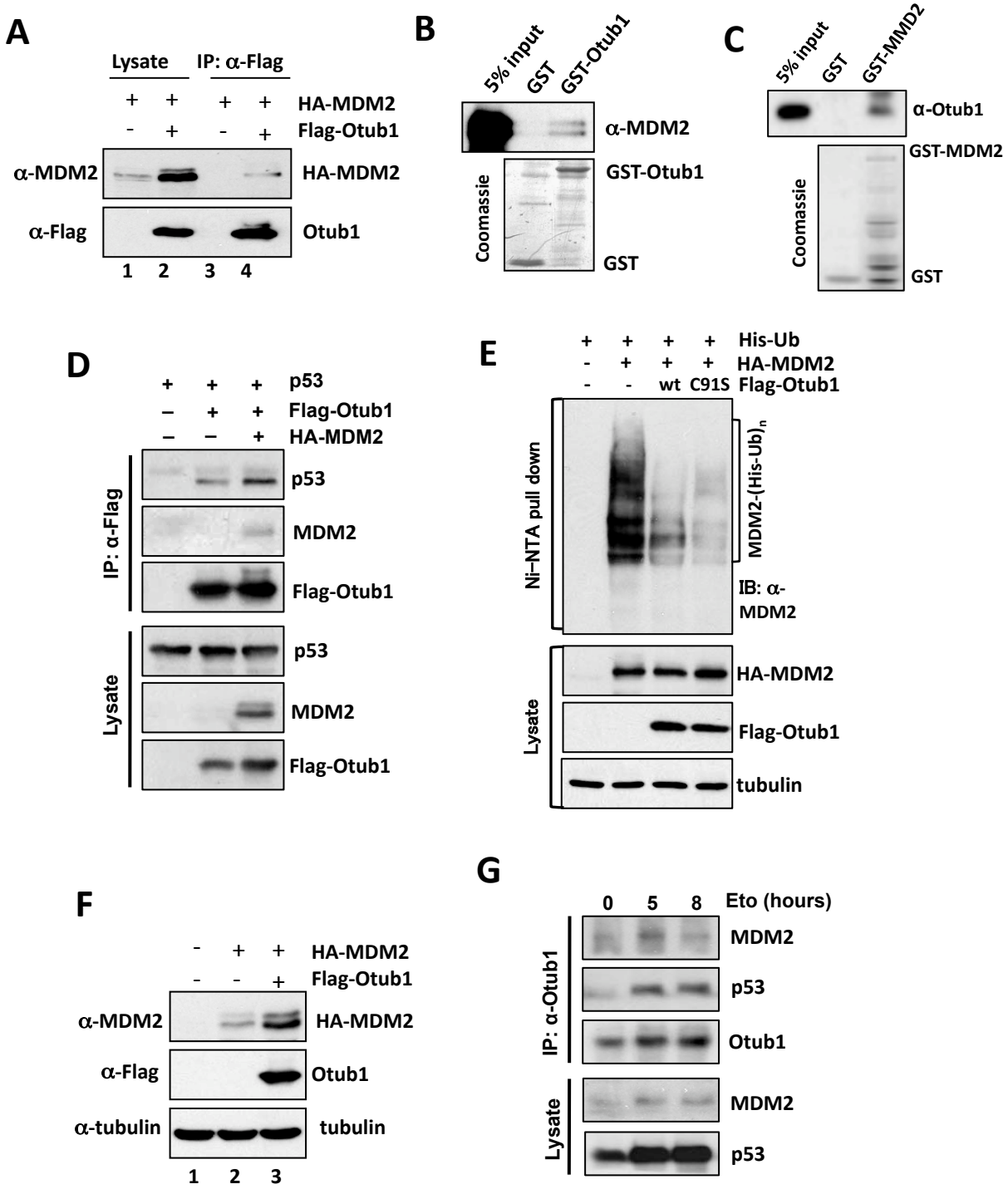
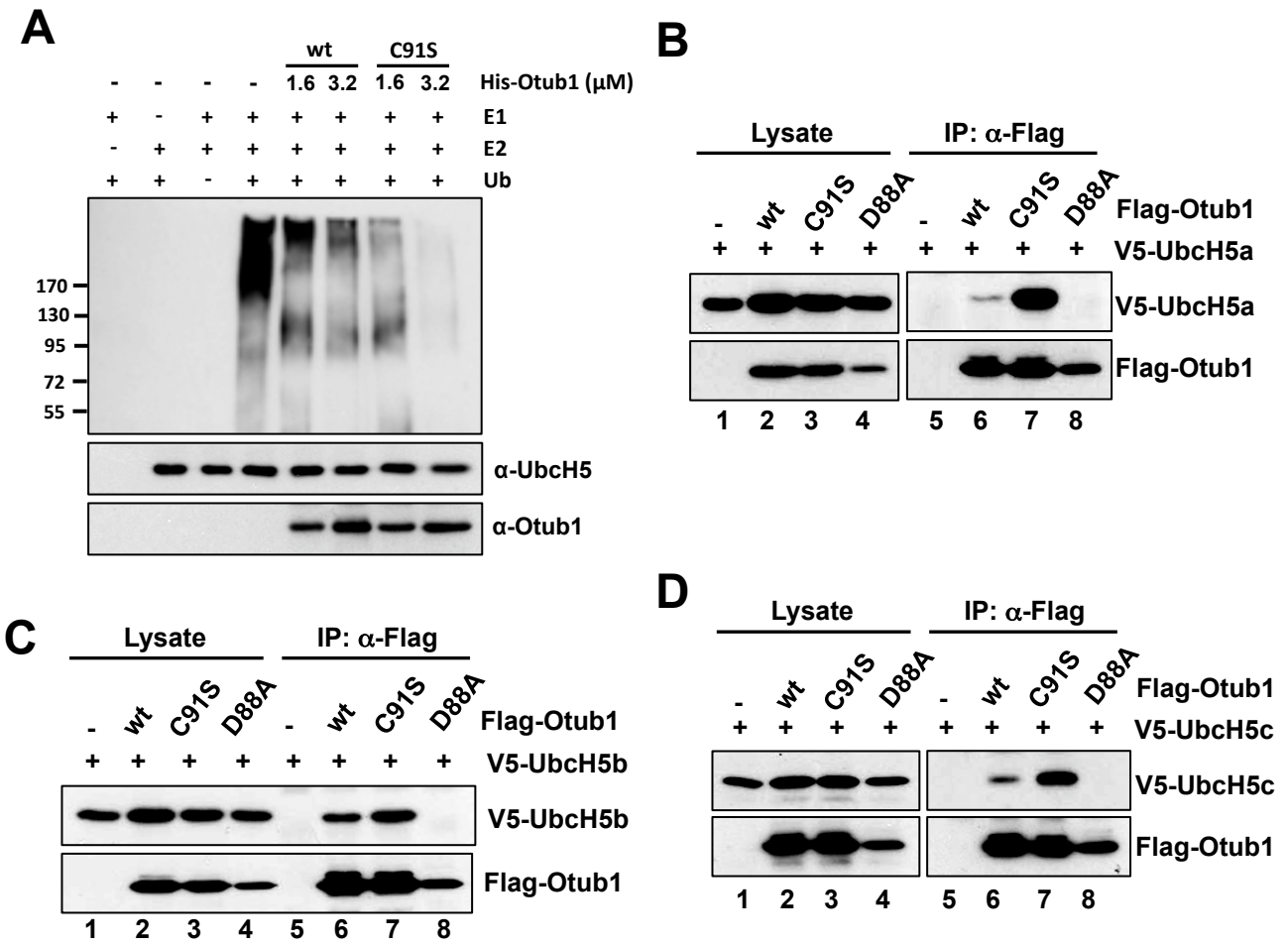


Supplementary Figure S7



Supplementary Figure S8